USP19-deubiquitinating enzyme regulates levels of major myofibrillar proteins in L6 muscle cells

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Sundaram P, Pang Z, Miao M, Yu L, Wing SS. USP19-deubiquitinating enzyme regulates levels of major myofibrillar proteins in L6 muscle cells. Am J Physiol Endocrinol Metab 297: E1283–E1290, 2009. First published September 22, 2009; doi:10.1152/ajpendo.00409.2009.—The ubiquitin-proteasome system plays an important role in the degradation of myofibrillar proteins that occurs in muscle wasting. Many studies have demonstrated the importance of enzymes mediating conjugation of ubiquitin. However, little is known about the role of deubiquitinating enzymes. We previously showed that the USP19-deubiquitinating enzyme is induced in atrophying skeletal muscle (Combaret L, Adegoke OA, Bedar N, Baracos V, Attax D, Wing SS. Am J Physiol Endocrinol Metab 288: E693–E700, 2005). To further explore the role of USP19, we used small interfering RNA (siRNA) in L6 muscle cells. Lowering USP19 by 70–90% in myotubes resulted in a 20% decrease in the rate of proteolysis and an 18% decrease in the rate of protein synthesis, with no net change in protein content. Despite the decrease in overall synthesis, there were ~1.5-fold increases in protein levels of myosin heavy chain (MHC), actin, and troponin T and a ~2.5-fold increase in troponymosin. USP19 depletion also increased MHC and tropomyosin mRNA levels, suggesting that this effect is due to increased transcription. Consistent with this, USP19 depletion increased myogenin protein and mRNA levels approximately twofold. Lowering myogenin using siRNA prevented the increase in MHC and tropomyosin upon USP19 depletion, indicating that myogenin mediated the increase in myofibrillar proteins. Dexamethasone treatment lowered MHC and increased USP19. Depletion of USP19 reversed the dexamethasone suppression of MHC. These studies demonstrate that USP19 modulates transcription of major myofibrillar proteins and indicate that the ubiquitin system not only mediates the increased protein breakdown but is also involved in the decreased protein synthesis in atrophying skeletal muscle.

ubiquitin-specific processing protease

SKELETAL MUSCLE IS THE MOST ABUNDANT TISSUE in the human body, accounting for approximately one-half of body weight. As a major site of metabolic activity, it directly converts chemical energy into mechanical energy and heat. It is also the largest reservoir of protein in the body. During starvation, muscle protein is degraded to provide amino acids for energy and for gluconeogenesis to maintain blood glucose. Muscle protein catabolism also occurs in a variety of pathological conditions such as disuse, loss of innervation, cancer, sepsis, hypercortisolism, and uncontrolled diabetes mellitus and results in significant morbidity (reviewed in Ref. 25). Therefore, much attention has been given to elucidating the biochemical mechanisms involved in mediating protein catabolism in muscle. These studies have shown that ubiquitin-proteasome-dependent proteolysis plays an important role in this process (reviewed in Ref. 1).

The highly conserved 76-amino acid peptide ubiquitin is expressed in all eukaryotic cells and exerts its cellular functions through its ability to be conjugated to other proteins. This conjugation is catalyzed sequentially by a cascade of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3) (reviewed in Ref. 35). When conjugated as a chain of more than four ubiquitin moieties, it can target the attached protein to the proteasome for degradation (41). Previous studies have shown that, in various catabolic conditions, steady-state levels of ubiquitinated proteins increase in the atrophying muscles (2, 26, 29, 42, 47). This suggests that there is an activation of ubiquitination that increases flux through the ubiquitin-proteasome pathway by increasing availability of ubiquitinated substrates for the proteasome. Supporting this model are many studies that have shown increased expression of many components involved in ubiquitin conjugation in various forms of muscle wasting (reviewed in Ref. 44). Increased expression in skeletal muscle of polylubiquitin genes (2, 4, 27, 40, 43, 46), ubiquitin-conjugating enzymes E214k, UBC-E2G, and UBC4 (7, 28, 40, 45), and ubiquitin protein ligases atrogin-1 (MAFbx), muscle RING finger-1 (MURF-1) (3, 14), and UBR2 (22) has been described in catabolic conditions. Increased expression of proteasome subunits (2, 5, 28, 36) has also been seen. Solid evidence for a role of the ubiquitin proteolytic system was confirmed with studies demonstrating that genetic inactivation in mice of the E3s MURF-1 or MAFbx/atrogen-1 (3) or of the ubiquitin-binding protein ZNF216 (18) leads to decreased loss of muscle mass following denervation.

Up to now, most of the studies of the ubiquitin system in muscle wasting have focused on the enzymes involved in the conjugation of ubiquitin to proteins. However, ubiquitination can also be modulated by deubiquitinating enzymes (reviewed in Ref. 38), and therefore, these may also be involved in the wasting process (44). Structurally, deubiquitinating enzymes can be divided broadly on the basis of sequence homology into five classes (reviewed in Ref. 33). The largest class (~75 in the human genome) are the ubiquitin-speciﬁc processing proteases (USPs). USPs are recognized by the presence of a core catalytic domain of ~450 amino acids delimited by cysteine and histidine boxes. Surrounding sequences are more divergent and are likely involved in mediating substrate speciﬁcity and other protein interactions. To date, only two deubiquitinating enzymes, USP14 and USP19, have been observed to be regulated in atrophying muscle, and both were induced rather than suppressed, suggesting that they do not act as negative regu-
Materials and Methods

Cell culture and transfection. L6 rat muscle cells (Dr. Amira Klip, University of Toronto) were grown at 37°C with 5% CO2 in α-MEM (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were induced to differentiate by replacing the medium with differentiation medium containing 100 nM CTL and 25 nM myogenin siRNA oligonucleotides. Three hours posttransfection, the transfection mixture was supplemented with an equal volume of α-MEM containing 20% fetal bovine serum, and the cells were grown overnight at 37°C. Some samples were harvested 24 h posttransfection (day 0), whereas the remaining cells were induced to differentiate by incubating the cells in differentiation medium. Samples were harvested at indicated time points up to day 4 of differentiation. The differentiation medium was changed every other day. To induce catabolism in the myotubes, the synthetic glucocorticoid dexamethasone (Sigma-Aldrich), actin (A4700; Sigma Aldrich), troponin (Sigma Aldrich), or tropomyosin (CH1; Developmental Studies Hybridoma Bank) were added to the cell culture medium at various time points (23). Twenty-five milligrams of protein were probed for USP19 (30), myogenin, myogenin, myogenin, and tropomyosin cDNA templates corresponding to nucleotide fragments 323–774, 323–774, 323–774, and 323–774, respectively. The reverse primer sequences were used for the PCR: myogenin, 5’-GCA-GAAGTGGTGGGCTCTGACAC-3’; MHC (2X isoform), 5’-GGT-CACCTTCCCTGTGGAG-3’ and tropomyosin 5’-AATGCTTTCAAGGTCTGA-3’.

Results

Depletion of USP19 modestly decreases the overall rates of protein synthesis and degradation. Since USP19 expression was previously observed to be closely but inversely correlated with muscle mass in rodents (10), we determined the effects of loss of USP19 on rates of protein turnover in L6 myotubes (Fig. 1). L6 myoblasts were transfected with USP19 or a nonspecific control siRNA and induced to differentiate into myotubes for 4 days. As was previously observed in rat fibroblasts (30), the USP19 siRNA oligonucleotides were effective, lowering USP19 levels by 70–90% (Fig. 2A). USP19 siRNA reduced the rate of incorporation of [3H]tyrosine into protein by 18% compared with control siRNA. We also measured the rate of protein turnover by the release of acid-soluble radioactivity from cells that had proteins prelabeled for 48 h with [3H]tyrosine. The rate of protein turnover was 20% lower.
Depletion of USP19 increases the expression of the myogenic regulatory factor myogenin. The increase in a broad range of myofibrillar proteins suggested that USP19 may be modulating a general transcriptional pathway that controls the expression of myofibrillar proteins. Myogenesis is dependent on a family of myogenic regulatory factors consisting of MyoD, myogenin, Myf5, and Myf6 (Mrf4) (reviewed in Ref. 34). MyoD is not expressed in L6 cells, and Myf6 is expressed only in terminal stages of differentiation that are not recapitulated in the in vitro system (11). Therefore, we tested the effect of depletion of USP19 on myogenin and Myf5, which are expressed in L6 cells (Fig. 4). Transfection of the cells with USP19 siRNA oligonucleotides did not affect Myf5 protein levels. However, myogenin protein levels were approximately twofold higher in cells depleted of USP19 on day 1, and they remained high in these cells until day 4, at which time they returned toward control levels. (Fig. 4, A and B). This was despite persistent low levels of USP19 on day 4 and suggests that other adaptations occur to perhaps limit differentiation to an optimal level of maturation. The overall increase in myogenin expression was associated with a slight but statistically significant increase in the extent of muscle cell fusion (defined as %cells containing 2 or more nuclei): CTL siRNA, 74 ± 2.9%; USP19 siRNA, 86 ± 1.7%.

To determine whether the increased myogenin protein levels in USP19-depleted cells were due to an increase in the levels of myogenin transcript, Northern blots were performed on RNA isolated from cells collected from day 0 to day 3 (Fig. 4C). USP19 siRNA resulted in an approximately twofold increase in myogenin mRNA on day 1 compared with CTL-transfected cells, and this persisted to day 3 (Fig. 4D). Taken together, these results indicate that USP19 regulates the expression of the myogenic regulatory factor myogenin.

USP19 regulates the expression of muscle-specific genes in a myogenin-dependent manner. To test whether USP19 depletion increases the expression of myofibrillar proteins through the increased expression of myogenin, we determined the effect of lowering myogenin levels on the ability of USP19 to modulate expression of MHC and tropomyosin (Fig. 5). We selected an siRNA oligonucleotide that would not silence myogenin expression completely but would, upon concomitant treatment with myogenin and USP19 siRNA oligonucleotides, result in myogenin levels similar to levels in control siRNA-transfected cells. As described above, silencing of USP19 increased levels of myogenin (Fig. 5A), MHC (Fig. 5, B and C), and tropomyosin (Fig. 5, B and D). However, these increases were no longer seen when myogenin was simultaneously depleted (Fig. 5, B–D). These results argue that USP19 does indeed act through myogenin to negatively regulate the expression of major muscle proteins.

Depletion of USP19 can inhibit the loss of MHC induced by dexamethasone. Excess glucocorticoids are well known to activate muscle proteolysis and appear to have a preferential

Fig. 1. Depletion of ubiquitin-specific processing protease-19 (USP19) in L6 myotubes slightly decreases both rates of protein synthesis and degradation. A: L6 cells were transfected with either control (CTL) or USP19 small interfering RNA (siRNA) oligonucleotides. After 4 days in differentiation medium, the cells were incubated with [3H]tyrosine. Two hours later protein was isolated, and rates of incorporation of radioactive amino acid into protein were measured (shown are means ± SE; n = 4; means are significantly different, P < 0.005). B: L6 cells were transfected with either CTL or USP19 siRNA oligonucleotides. After 1 day in differentiation medium, the cells were incubated with medium containing [3H]tyrosine. Two days later, the medium was changed and the rate of release of free radiolabeled tyrosine into the medium measured at the indicated time points. Shown are means ± SE; n = 4 and the rate equations obtained by linear regression analysis. CTL significantly different from USP19. P < 0.025 by 2-way ANOVA.

in cells transfected with USP19 siRNA than in control transfected cells. The similar decreases in the rates of protein synthesis and degradation indicate that overall protein turnover was probably not affected, and indeed, total protein content was unchanged by USP19 siRNA (data not shown).

Depletion of USP19 increases the expression of major myofibrillar proteins. Although depletion of USP19 did not affect overall protein turnover, there could still be specific effects on myofibrillar proteins, which, although they are the dominant proteins in differentiated muscle, are minor proteins in myotubes in culture. To test this hypothesis, we determined how loss of USP19 affected levels of major myofibrillar proteins in these muscle cells. Myotubes were collected after 4 days of differentiation, and the cell lysates were analyzed by Western blotting (Fig. 2). Depletion of USP19 increased levels of MHC, actin, and troponin T 1.5-fold and tropomyosin two- to threefold. There were no changes in levels of a nonmyofibrillar cytoskeletal protein, tubulin, indicating that these effects were specific. These effects were observed with two independent well-characterized siRNA oligonucleotides (30), indicating that this was unlikely to be due to off-target effects. Since myofibrillar proteins are known to have very long half-lives [e.g., MHC half-life of 27 h (16)], these effects are likely due to increased synthesis of these proteins. Indeed, depletion of USP19 also increased levels of mRNA transcripts of MHC (Fig. 3, A and C) and tropomyosin (Figs. 3, B and D), suggesting that USP19 acts at the level of gene transcription to increase the expression of these major muscle proteins.
effect on myofibrillar proteins (20). We observed previously that USP19 expression increases in catabolic conditions, including dexamethasone treatment (10). Other investigators have reported that incubation of L6 myotubes with dexamethasone can increase overall protein breakdown (32) and lower levels of MHC (13). Therefore, we tested whether dexamethasone can induce USP19 in muscle cells and whether lowering USP19 in muscle cells can reverse the loss of MHC induced by this catabolic stimulus (Fig. 6). We observed a small (~20%) but reproducible increase in USP19 levels in the dexamethasone-treated myotubes and confirmed that dexamethasone is able to lower MHC levels in L6 myotubes (Fig. 6, A and C). Silencing of USP19 in these muscle cells was indeed able to reverse the loss of MHC protein induced by dexamethasone to levels similar to cells treated with control siRNA but not to the higher levels seen with USP19 siRNA alone (Fig. 6, A and B).

**DISCUSSION**

We have demonstrated for the first time that a deubiquitinating enzyme can regulate the expression of a panel of major myofibrillar proteins, including MHC, actin, tropomyosin, and troponin. Loss of USP19 in L6 myotubes increased the level of these proteins (Fig. 2). Although it is known that myofibrillar proteins such as MHC, myosin light chains (8, 9), and troponin I (21) are ubiquitinated in muscle, these myofibrillar proteins do not appear to be direct substrates of USP19, because siRNA-mediated loss of this deubiquitinating activity would have increased their ubiquitination and degradation and therefore lowered rather than raised their levels. Instead, our data argue that USP19 negatively regulates a transcriptional program of muscle-specific genes. mRNA levels of MHC and tropomyosin were found to be elevated upon USP19 depletion.
Myogenic regulatory factors are a group of muscle-specific transcription factors that are master regulators of skeletal muscle development (34). We found for the first time that USP19 can modulate transcription of myogenin, a myogenic regulatory factor member that is increased during muscle differentiation, because silencing of USP19 increased levels of both myogenin mRNA and protein (Fig. 4). However, USP19 does not necessarily act at the beginning of the differentiation process, because similar increases in expression of MHC could be observed when L6 cells were transfected with USP19 siRNA oligonucleotides on day 2 of differentiation and harvested on day 4 (data not shown). In addition, the increase in myogenin was associated with only a small, 15% increase in the number of myotubes, suggesting that most of the stimulus of myogenic differentiation is achieved with myogenin levels present in the cells transfected with the control oligonucleotides. The mechanism by which USP19 regulates myogenin remains unclear. The simplest model is that a transcriptional repressor of myogenin transcription is a substrate of USP19 and can be destabilized upon silencing of USP19. However, at this time, we cannot exclude the possibility that USP19 acts on substrate(s) upstream in the signaling pathways that modulate the synthesis of myofibrillar proteins.

Silencing of USP19 did lower the rate of overall protein degradation by 20%, and we cannot exclude that this small change contributes to the overall increase in steady-state levels of myogenin.
of myofibrillar proteins. However, in view of the previously measured half-life of 27 h for MHC in myotubes (16), USP19 siRNA would have to prolong this half-life markedly to induce a 50% increase in steady-state levels. In addition, the fact that preventing the increase in myogenin expression completely blocks the increase in myofibrillar proteins (Fig. 5) also argues that proteolysis does not play a major role in the regulation of myofibrillar protein levels by USP19.

Fig. 5. USP19 inhibits the expression of muscle-specific genes in a myogenin-dependent manner. L6 myoblasts were transfected either singly with nonspecific CTL, USP19 (no. 7), or myogenin siRNA or doubly with both myogenin and USP19 siRNA oligonucleotides and induced to differentiate for 3 days. Cell lysates were analyzed by Western blotting for myogenin and USP19 (A) and MHC and tropomyosin (B). Effects of single and double siRNA-mediated depletion on tropomyosin and MHC proteins were quantified in C and D, respectively (n = 4). *P < 0.05 compared with CTL (1-way ANOVA).

Fig. 6. Depletion of USP19 can reverse dexamethasone-induced loss of MHC. L6 myoblasts were transfected with nonspecific control (CTL) or USP19 siRNA (no. 7) oligonucleotides. On day 2 of differentiation, the developing myotubes were treated with 10 μM dexamethasone or vehicle. Two days later, the cells were harvested and the lysates analyzed by Western blotting for the indicated proteins. A: representative blots. B and C: quantitation of MHC and USP19 protein levels (n = 3). Shown are means ± SE. *Means that are statistically significant with P < 0.05 (1-way ANOVA).
As mentioned above, a study using gene arrays to identify mRNA transcripts regulated in various conditions of muscle wasting identified USP14 as being induced in wasting conditions (24). This proteasome-associated deubiquitinating enzyme is orthologous to yeast UBP6, and genetic manipulation in yeast indicates that it inhibits protein degradation by deubiquitinating substrates and inhibiting proteasome activity (17). Thus, in contrast to our observations on USP19 reported here, the role of induction of this enzyme in conditions of muscle atrophy remains unclear.

In our previous study, USP19 mRNA levels increased by 30–200% in rat skeletal muscle atrophying in response to cancer, glucocorticoids, fasting, or diabetes. During refeeding, the increased mRNA levels of USP19 in the fasted state returned to normal, coinciding with the recovery of muscle mass (10). Together with our loss of USP19 function in studies in cultured cells reported here, this suggests that USP19 has a specific role in regulating levels of myofibrillar proteins in adult differentiated muscle. Although myogenin levels are induced in adult myofibers upon denervation, studies involving other forms of muscle wasting have reported lower levels of myogenin expression in atrophying muscle (12, 37), suggesting that the mechanism that we have identified in cultured muscle cells may be relevant in vivo.

Steady-state levels of muscle proteins are determined by the balance between their rates of synthesis and degradation. In many catabolic conditions, muscle atrophy arises from both a fall in the rate of synthesis and an activation of the rate of degradation (reviewed in Ref. 19). A large body of previous work has shown that components of the ubiquitin-conjugating apparatus are induced in atrophying muscles, indicating that the ubiquitin-proteasome pathway contributes to the increase in muscle protein degradation (44). A recent report indicates that the induction of the MAFb3 ligase may also contribute to inhibition of protein synthesis by targeting the eIF3 initiation factor for degradation (23). To our knowledge, we are showing for the first time evidence for an important role for a deubiquitinating enzyme in muscle atrophy and evidence that this enzyme may contribute to wasting by inhibiting the synthesis of major myofibrillar proteins. Our results thus expand the functions of the ubiquitin system in muscle physiology. Consistent with a potentially important role of USP19 in pathological conditions of muscle wasting, we found that depletion of USP19 reversed the loss of MHC induced by dexamethasone in L6 myotubes and that this catabolic stimulus can induce expression of USP19 (Fig. 6). Transfection with USP19 siRNA in the presence of dexamethasone did not increase MHC levels to levels seen with transfection of USP19 siRNA alone. This suggests that USP19 depletion cannot reverse all of the effects of glucocorticoids on MHC. Indeed, glucocorticoids promote muscle catabolism through multiple mechanisms, including decreased insulin and IGF-I signaling, increased myostatin expression, decreased translation, and activation of transcription factors that lead to induction of ubiquitin protein ligases (reviewed in Ref. 31). Therefore, our results are consistent with USP19 modulating glucocorticoid effects specifically on the transcriptional pathway that leads to synthesis of myofibrillar proteins. At this time, we cannot exclude the possibility that the siRNA and glucocorticoid effects on MHC are independent of each other and additive. Further exploration of this new mechanism of muscle wasting will require the study of USP19-deficient mice. Nonetheless, our present findings suggest that inhibition of USP19 could be a novel therapeutic mechanism for the prevention and treatment of muscle protein catabolism.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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